FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **217301US0PCT**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR): **10/004782**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/03932	**15 JUNE 2000**	**17 JUNE 1999**

TITLE OF INVENTION

TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING D-AMINOACYLASE

APPLICANT(S) FOR DO/EO/US

Ken-ichi TAKEUCHI, et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Notice of Priority / PCT/IB/304 / PCT/IB/308
PTO-1449 / Drawings (2 sheets)
Sequence Listing (5 sheets)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/009782	PCT/JP00/03932	217301US0PCT

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00	
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00	
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00	
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00	
	ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00
	Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	$130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	4 - 20 =	0	x $18.00	$0.00
Independent claims	2 - 3 =	0	x $84.00	$0.00
Multiple Dependent Claims **(check if applicable)**.			☐	$0.00

TOTAL OF ABOVE CALCULATIONS =	$1,020.00
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	$0.00
SUBTOTAL =	$1,020.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	$0.00
TOTAL NATIONAL FEE =	$1,020.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	$0.00
TOTAL FEES ENCLOSED =	$1,020.00
Amount to be: refunded	$
charged	$

a. ☒ A check in the amount of **$1,020.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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Dec. 17 2001
DATE

217301US-0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF :

KEN-ICHI TAKEUCHI ET AL : ATTN: APPLICATION DIVISION

SERIAL NO: 10/009,782 :

FILED: DECEMBER 17, 2001 :

FOR: TRANSFORMED MICROORGANISM: AND PROCESS FOR PRODUCING D-AMINOACYLASE

SECOND PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE CLAIMS

Please add the following new claims.

5. (New) The transformed microorganism according to claim 1, wherein the D-aminoacylase-producing gene is obtained from Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain.

6. (New) The transformed microorganism according to claim 1, wherein the D-aminoacylase-producing gene is modified by designing a specific nucleotide sequence (GAAGGA) in the ribosome-binding site and inserting the nucleotide sequence in the position of the ninth base upstream of the translation initiation point of the gene.

7. (New) The transformed microorganism according to claim 1, wherein the D-aminoacylase-producing gene is modified by creating a HindIII recognition site of Escherichia coli in the upstream and downstream of the gene, purifying and excising the resulting gene and ligating the gene into an expression vector.

8. (New) The transformed microorganism according to claim 1, wherein the zinc tolerance of the host microorganism is such that the cell weight of the microorganism either increases, or decreases within a range of 10% in a culture medium with 2 mM zinc added thereto on the basis of the cell weight (A660 nm) in a zinc-free culture medium.

9. (New) The transformed microorganism according to claim 1, wherein the zinc tolerance of the host microorganism is such that the cell weight of the microorganism either increases, or decreases within a range of 20% in a culture medium with 5 mM zinc added thereto on the basis of the cell weight (A660 nm) in a zinc-free culture medium.

10. (New) The transformed microorganism according to claim 1, wherein the host microorganism is Escherichia coli.

11. (New) The process for producing D-aminoacylase according to claim 3, wherein the culture medium is a nutritious culture medium containing a tac promotor-inducing substance as an inducer.

12. (New) The process for producing D-aminoacylase according to claim 11, wherein the inducer is isopropyl thiogalactoside (IPTG) or lactose.

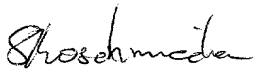
13. (New) The process for producing D-aminoacylase according to claim 12, wherein the concentration of lactose is adjusted to 0.1 to 1%.

REMARKS

Claims 1-13 are active in the present application. Claims 5-13 are new claims. Support for new Claim 5 is found on page 8. Support for new Claim 6 is found on page 5. Support for new Claim 7 is found on page 6. Support for new Claim 8 is found on page 6. Support for new Claim 9 is found on page 6. Support for new Claim 11 is found on page 8. Support for new Claim 12 is found on page 9. Support for new Claim 13 is found on page 9. No new matter is believed to have been added by this amendment. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

OBLON, SPIVAK, McCLELLAND,
MAIER & NEUSTADT, P.C.



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Marked-Up Copy
Serial No:

Amendment Filed on:
3-25-2002

IN THE CLAIMS

Claims 5-13 (New).

Docket No.: 217301US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

Ken-ichi TAKEUCHI et al : ATTN: BOX SEQUENCE

SERIAL NO. New U.S. Appln.
(Based on PCT/JP00/03932) :

FILED: HEREWITH :

FOR: TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING D-AMINOACYLASE

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as follows:

Page 17 (Abstract), after the last line, please delete the original Sequence Listing and replace with the substitute Sequence Listing appended hereto.

REMARKS

Applicants submit herewith a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. The sequence information recorded in the corresponding computer-readable Sequence Listing is identical to the paper copy of the substitute Sequence Listing. Support for all of the sequences listed in the substitute Sequence Listing is found in the present application as originally filed. No new matter is believed to have been introduced by the submission of the substitute Sequence Listing and the corresponding computer-readable Sequence Listing.

An action on the merits and allowance of the claims is solicited.

Respectfully submitted,

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Description

Title of the Invention

TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING D-AMINOACYLASE

Technical Field

The present invention relates to a transformed microorganism prepared by inserting into a zinc-tolerant microorganism a D-aminoacylase-producing gene which selectively produces D-aminoacylase alone between D-aminoacylase and L-aminoacylase, and a process for producing D-aminoacylase by utilizing the transformed microorganism.

Background Art

D-aminoacylase is an enzyme industrially useful for the production of D-amino acids of high optical purity, which are needed for uses in side chains of antibiotics, peptide drugs and the like.

Chemical and Pharmaceutical Bulletinn 26, 2698 (1978) discloses Pseudomonas sp. AAA6029 strain as a microorganism simultaneously producing D-aminoacylase and L-aminoacylase. Japanese Patent Application Laid-open No. Sho-53-59092 discloses actinomycetes such as Streptomyces olibaceus S•6245. The use of these microorganisms results in the simultaneous

production of the optical isomers, D-aminoacylase and L-aminoacylase, apart from the potency to produce D-aminoacylase. Thus, laborious and costly procedures are disadvantageously required for the separation of the two.

Alternatively, for example, Japanese Patent Application Laid-open No. Hei-1-5488 discloses Alcaligenes denitrificans subsp. xylosoxydans M1-4 strain as a microorganism selectively producing D-aminoacylase alone. In case that this bacterial strain is utilized, no laborious work is required for the separation of D-aminoacylase from L-aminoacylase. However, the potency of the bacterial strain to produce D-aminoacylase is insufficient. Furthermore, the nucleotide sequence of the D-aminoacylase-producing gene is not elucidated in Japanese Patent Application Laid-open No. Hei-1-5488. Thus, no modification of the gene so as to improve the D-aminoacylase-producing potency or no creation of a transformed bacterium with a high productivity has been accomplished.

Under such circumstances, the present inventors Moriguchi, et al. elucidated the structure of the D-aminoacylase-producing gene in the Alcaligenes xylosoxydans subsp. xylosoxydans A-6 strain and demonstrated its nucleotide sequence of SEQ ID NO: 1 in the sequence listing. Further, a certain genetic modification of the D-aminoacylase-producing gene successfully improved the D-aminoacylase-producing potency of the resulting transformed bacterium

(Protein Expression and Purification 7, 395-399 (1996)).

Disclosure of the Invention

The inventors' subsequent research works have elucidated that the D-aminoacylase-producing potencies of various transformed bacteria with the aforementioned D-aminoacylase-producing gene inserted therein are greatly improved in zinc ion-containing culture media. It has also been found that the producing potencies are prominently improved by controlling the zinc ion concentration within a predetermined range, in particular.

Furthermore, it has been found that the above-mentioned effect varies significantly depending on the type of a host microorganism and that a host microorganism with high such effect generally exerts zinc tolerance even prior to the transformation thereof. Herein, the zinc tolerance means that the growth potency of a bacterium as measured on the basis of the cell weight (A660 nm) is hardly inhibited by the addition of zinc ion.

The findings mentioned above indicate the followings (1) and (2). (1) The expression of a transformed microorganism with a D-aminoacylase-producing gene of SEQ ID NO: 1 in the sequence listing is enhanced in the presence of a given quantity of zinc ion, though the reason has not been elucidated. (2) Since it is believed that zinc ion functions in an inhibiting

manner on common microorganisms, a congenitally zinc tolerant microorganism should be selected as a host to insert the gene therein so as to sufficiently procure the effect of zinc ion.

Based on the above-mentioned points, the invention provides a microorganism transformed with a D-aminoacylase-producing gene, the D-aminoacylase-producing potency of which can be enhanced far more greatly with the addition of zinc ion to a culture medium therefor. The invention further provides a process for producing D-aminoacylase using the transformed microorganism.

The transformed microorganism of the invention is a microorganism having aquired high-expression ability to produce D-aminoacylase in a zinc ion-containing culture medium, prepared by inserting into a host microorganism with zinc tolerance a D-aminoacylase-producing gene the expression of a gene product of which is enhanced in the presence of zinc ion. The transformed microorganism is a microorganism transformed with a D-aminoacylase-producing gene, and due to the addition of zinc ion to the culture medium, the D-aminoacylase-producing potency thereof can be enhanced to maximum.

In the transformed microorganism of the invention, the D-aminoacylase-producing gene more preferably has a nucleotide sequence of SEQ ID NO: 1 in the sequence listing or a nucleotide sequence hybridizing to the nucleotide sequence

of SEQ ID NO: 1 in the sequence listing under stringent conditions and effectively encoding D-aminoacylase. It has been confirmed that a D-aminoacylase-producing gene having a nucleotide sequence of SEQ ID NO: 1 in the sequence listing is a gene the expression of a gene product of which can greatly be enhanced in the presence of zinc ion. Further, a gene of a nucleotide sequence hybridizing to the nucleotide sequence of SEQ ID NO: 1 in the sequence listing under stringent conditions and effectively encoding D-aminoacylase can be expected to have similar characteristics.

More preferably, in the transformed microorganism of the invention, a host microorganism is Escherichia coli. It has been confirmed that Escherichia coli has zinc tolerance. Further, the mycological and physiological properties, culture conditions and maintenance conditions of Escherichia coli are well known. Thus, the production of D-aminoacylase at high efficiency can be done under readily controllable conditions.

Still more preferably, in the transformed microorganism of the invention, a D-aminoacylase-producing gene which is to be inserted into a host microorganism is subjected to the following modification (1) and/or (2). (1) Modification for improving the translation efficiency, comprising designing a specific nucleotide sequence (GAAGGA) in the ribosome-binding site and inserting the nucleotide sequence in the position of

the ninth base upstream of the translation initiation point of the gene. This modification improves the translation efficiency of the D-aminoacylase-producing gene. (2) Modification for improving the gene expression efficiency, comprising creating a HindIII recognition site of Escherichia coli in the upstream and downstream of the gene, subsequently purifying and excising the resulting gene and ligating the gene into an expression vector. This modification improves the expression efficiency of the D-aminoacylase-producing gene.

A zinc-tolerant microorganism is used as a host microorganism for obtaining a transformed microorganism in accordance with the invention. More specifically, a microorganism should be used, the growth potency of which in culture media, as measured on the basis of increase or decrease of the cell weight (A660 nm), is not so much inhibited by the addition of zinc ion. One of the standards to evaluate zinc tolerance is as follows. On the basis of the cell weight (A660 nm) of the microorganism in a zinc-free culture medium, the cell weight in the same culture medium under the same conditions except for the addition of 2 mM zinc either increases, or decreases within a range of 10 %. Otherwise, the above-mentioned cell weight in the same culture medium under the same conditions except for the addition of 5 mM zinc increases, or decreases within a range of 20 %.

Although the taxonomical group of the host microorganism

is not limited, it is generally preferable to use such host microorganisms that the morphological and physiological properties are well known and the culture conditions and maintenance conditions are also well known. A preferable example of such a host microorganism is Escherichia coli. Compared with Escherichia coli, microorganisms of the species Alcaligenes xylosoxidans including A-6 strain do not have zinc tolerance.

The means for inserting a D-aminoacylase-producing gene into a host microorganism is not specifically limited. For example, an insertion method comprising plasmid ligation, an insertion method comprising ligation to bacteriophage DNA, and the like may be arbitrarily selected as required.

The D-aminoacylase-producing gene in accordance with the invention is a gene selectively producing D-aminoacylase alone between D-aminoacylase and L-aminoacylase, and is of a type in which the activity expression is enhanced in the presence of zinc ion in the culture medium. As a preferable example of such D-aminoacylase-producing gene, the gene with the nucleotide sequence of SEQ ID NO: 1 in the sequence listing has been confirmed. Further, genes of nucleotide sequences hybridizing to the nucleotide sequence of SEQ ID NO: 1 in the sequence listing under stringent conditions and effectively encoding D-aminoacylase are also preferable, except for genes which do not actually enhance the activity expression with zinc

ion in the culture medium.

The D-aminoacylase-producing gene with the nucleotide sequence of SEQ ID NO: 1 was obtained from the Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain. The A-6 strain is a D-aminoacylase-producing strain obtained from soil in nature via screening.

The process for producing D-aminoacylase in accordance with the invention comprises culturing any transformed microorganism as described above in a culture medium containing zinc ion, and obtaining D-aminoacylase from the culture. Zinc ion can be provided by adding an appropriate amount of zinc compounds such as zinc chloride and zinc sulfate to the culture medium. This process enables to produce D-aminoacylase at a high efficiency.

In the process for producing D-aminoacylase in accordance with the invention, the concentration of zinc ion contained in the culture medium is preferably controlled to 0.1 to 10 mM. This process enables to optimize the zinc ion concentration in the culture medium, and to produce D-aminoacylase at a particularly high efficiency.

In the process for producing D-aminoacylase, other procedures and conditions for carrying out the process are not specifically limited. Nevertheless, the culture is preferably carried out in a nutritious culture medium containing tac promoter-inducing substances (for example, isopropyl

thiogalactoside (IPTG), lactose and the like) as inducers. Further, the concentration of lactose then is preferably adjusted to about 0.1 to 1 %.

Brief Description of the Drawings

Fig. 1 schematically depicts the plasmid used for ligating with the D-aminoacylase-producing gene. Fig. 2 schematically depicts the plasmid ligated with the D-aminoacylase-producing gene.

Best Mode for Carrying out the Invention

Best modes for carrying out the invention are described below together with comparative example. The invention is never limited to these modes for carrying out the invention.

(Obtainment of gene and determination of nucleotide sequence)

The chromosomal DNA obtained from Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain was partially digested with restriction endonuclease Sau3AI, to obtain by fractionation DNA fragments of 2 to 9 Kb. The resulting DNA fragments were inserted in and ligated at the BamHI recognition site of a known plasmid pUC118. Escherichia coli JM109 was transformed with the ligated plasmid, to obtain an ampicillin-resistant transformant strain. Among the thus obtained transformant strains, a strain with a potency of selectively producing D-aminoacylase alone was obtained. The

transformant strain with the potency retained the plasmid with a 5.8-Kb insert fragment.

The 5.8-Kb insert fragment in the plasmid was trimmed down to deduce the position of the D-aminoacylase-producing gene. According to general methods, then, the nucleotide sequence as shown in SEQ ID NO:1 in the sequence listing was determined for the DNA of about 2.0 Kb. An amino acid sequence corresponding to the nucleotide sequence is also shown in the sequence listing. Consequently, an open reading frame (ORF) consisting of 1452 nucleotides starting from ATG was confirmed. (Gene modification)

From the plasmid with the 5.8-Kb insert fragment was excised a 4-Kb DNA fragment via BamHI-HindIII digestion, which was then ligated into a known plasmid pUC118 to construct a ligated plasmid pAND118. Using the resulting plasmid, site-directed mutagenesis using primers was effected, to thereby prepare a ribosome-binding site (RBS)-modified plasmid pANSD1.

Using the plasmid pANSD1 as template, site-directed mutagenesis using primers was effected, thereby to prepare a plasmid pANSD1HE having an EcoRI recognition site and a HindIII recognition site immediately upstream the RBS and immediately downstream the ORF, respectively.

Then, the plasmid pANSD1HE was digested with restriction endonucleases EcoRI and HindIII to prepare a 1.8-Kb DNA

fragment, which was inserted in and ligated at the EcoRI-HindIII site in the plasmid pKK223-3 shown in Fig. 1 to obtain the plasmid pKNSD2 shown in Fig. 2.

(Transformed Escherichia coli)

The plasmid DNA was inserted into a host strain derived from the Escherichia coli K-12 strain by the D. HANAHAN's method (DNA Cloning, Vol.1, 109-136, 1985), thereby to obtain a transformed Escherichia coli (E. coli) TG1/pKNSD2.

(Zinc tolerance of bacterial strain as gene source)

The Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain was cultured at 30°C for 24 hours in a culture medium (pH 7.2, zinc-free) containing 0.2 % potassium dihydrogen phosphate, 0.2 % dipotassium hydrogen phosphate, 2 % polypeptone, 0.01 % magnesium sulfate and 1 % glycerin, and in culture media of the same composition but with addition of zinc oxide to concentrations 0.2 mM, 2.0 mM and 5.0 mM, respectively. After culturing, the cell weight (A660 nm) was measured to evaluate the zinc tolerance. Then, the pH of the culture media after culturing was measured. The results are shown in the column of "A-6 bacteria" in Table 1.

Table 1

Microbial strain	Zinc concentration (mM)	Post-culture pH	Cell weight (A660)	Relative value (%)
A-6 bacteria	0.0	7.58	8.09	100.0
	0.2	7.62	7.75	95.8
	2.0	7.56	5.23	64.6
	5.0	7.68	3.34	41.3

TG1 (host bacterium)	0.0	5.01	5.68	100.0
	0.2	4.99	5.93	104.4
	2.0	4.98	5.55	97.7
	5.0	5.01	4.98	87.7
pKNSD2/TG1 (recombinant bacterium)	0.0	5.00	6.45	100.0
	0.2	5.01	6.70	103.9
	2.0	4.98	6.09	94.4
	5.0	5.01	5.47	84.8

Table 1 shows that the cell weight of the A-6 strain in the zinc-added culture media was greatly decreased (decreased by about 35 % in the 2.0 mM zinc-added culture medium and by about 60 % in the 5.0 mM zinc-added culture medium), compared with the cell weight of the A-6 strain in the zinc-free culture medium. This indicates that the A-6 strain was not zinc-tolerant.

(Zinc tolerance of host bacterium)

The zinc tolerance of the strain derived from the Escherichia coli K-12 strain used as the host bacterium was examined, using a culture medium of the same composition as for the A-6 strain, by measuring the cell weight (A660 nm) in the same manner. The results are shown in the column of "TG1 (host bacterium)".

Table 1 shows that the cell weight of the host bacterium in the zinc-added culture media was not so greatly decreased (decreased by about 3 % in the 2.0 mM zinc-added culture medium and by about 12 % in the 5.0 mM zinc-added culture medium, and even increased in the 0.2 mM zinc-added culture medium),

compared with the cell weight of the host bacterium in the zinc-free culture medium. This indicates that the host bacterium was zinc-tolerant.

(Zinc tolerance of transformed Escherichia coli)

The zinc tolerance of the transformed Escherichia coli (E.coli) TG1/pKNSD2 was examined using a culture medium of the same composition as for the A-6 strain by measuring the cell weight (A660 nm) in the same manner. The results are shown in the column of "pKNSD2/TG1 (recombinant bacterium)".

Table 1 shows that the cell weight of the transformed bacterium in the zinc-added culture media was not so greatly decreased (decreased by about 5 % in the 2.0 mM zinc-added culture medium and by about 15 % in the 5.0 mM zinc-added culture medium, and even increased in the 0.2 mM zinc-added culture medium), compared with the cell weight of the transformed bacterium in the zinc-free culture medium. This indicates that the transformed Escherichia coli was zinc-tolerant.

(Effect of zinc addition on transformed Escherichia coli)

The transformed Escherichia coli (E. coli) TG1/pKNSD2 was pre-cultured in a culture medium (pH 7.0) containing 1 % bactotryptone, 0.5 % bacto-yeast extract, 0.5 % sodium chloride and 100 μg/ml ampicillin, at 30°C for 16 hours.

Subsequently, the post-preculture transformed Escherichia coli was cultured at 30°C for 24 hours in a culture medium (pH 7.0, zinc-free) containing 0.2 % potassium

dihydrogen phosphate, 0.2 % dipotassium hydrogen phosphate, 2 % polypeptone, 0.01 % magnesium sulfate, 1 % glycerin and 0.1 % lactose as an inducer, and culture media of the same composition but with addition of zinc oxide to concentrations 0.2 mM and 2.0 mM. Additionally, the broth-out pH of the culture broth as well as the enzyme activity (U/mL) of D-aminoacylase in the culture broth (A660 nm) was measured.

Consequently, the enzyme activity in the 0.2 mM zinc-added culture medium was 58.85 U/mL (broth-out pH of 5.03) and the enzyme activity in the 2.0 mM zinc-added culture medium was 109.79 U/mL (broth-out pH of 5.11), compared with the enzyme activity of 21.78 U/mL in the zinc-free culture medium (broth-out pH of 5.05). Thus, it has been confirmed that the addition of zinc ion, at least within a predetermined concentration range, greatly improves the D-aminoacylase-producing potency.

For comparison, additionally, the A-6 strain was pre-cultured in the culture medium for preculture (no ampicillin was however added) under the same conditions, and was then cultured in the culture medium of the same composition for culture, except for the change of the inducer from 0.1 % of lactose to 0.1 % of N-acetyl-D, L-leucine. Then, the broth-out pH of the culture broth as well as the enzyme activity (U/mL) of D-aminoacylase in the culture broth (A660 nm) was assayed.

Consequently, the enzyme activity in the 0.2 mM zinc-added culture medium was 0.12 U/mL (broth-out pH of 7.48) and the enzyme activity in the 2.0 mM zinc-added culture medium was 0.29 U/mL (broth-out pH of 7.43), compared with the enzyme activity of 0.29 U/mL in the zinc-free culture medium (broth-out pH of 7.47). Thus, no effect of zinc ion addition on the improvement of the D-aminoacylase-producing potency could be confirmed.

Industrial Applicability

As described above, D-aminoacylase, as an industrially useful enzyme, can be produced highly efficiently and selectively by using the transformed microorganism of the invention.

Claims

1. A transformed microorganism having acquired high-expression ability to produce D-aminoacylase in a zinc ion-containing culture medium, prepared by inserting into a host microorganism with zinc tolerance a D-aminoacylase-producing gene the expression of a gene product of which is enhanced in the presence of zinc ion.

2. The transformed microorganism according to claim 1, wherein the D-aminoacylase-producing gene has a nucleotide sequence of SEQ ID NO:1 in the sequence listing or a nucleotide sequence hybridizing to the nucleotide sequence of SEQ ID NO: 1 in the sequence listing under stringent conditions and effectively encoding D-aminoacylase.

3. A process for producing D-aminoacylase, comprising culturing in a culture medium containing zinc ion a transformed microorganism prepared by inserting into a host microorganism with zinc tolerance a D-aminoacylase-producing gene the expression of the gene product of which is enhanced in the presence of zinc ion, and obtaining D-aminoacylase from the culture.

4. The process for producing D-aminoacylase according to claim 3, wherein the concentration of zinc ion contained in the culture medium is controlled to 0.1 to 10 mM.

Abstract

A transformed microorganism prepared by inserting into a host microorganism with zinc tolerance a D-aminoacylase-producing gene which selectively produces D-aminoacylase alone between D-aminoacylase and L-aminoacylase. A process comprising culturing the transformed microorganism in a culture medium containing zinc ion and obtaining D-aminoacylase from the culture at a high efficiency.

FIG.1

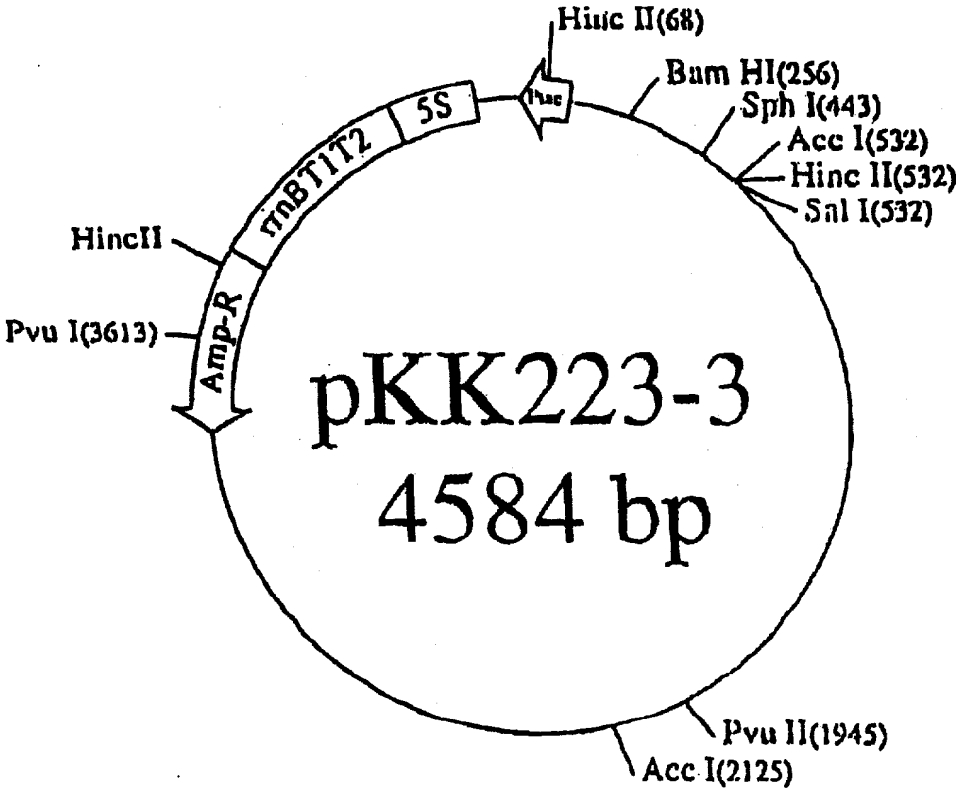
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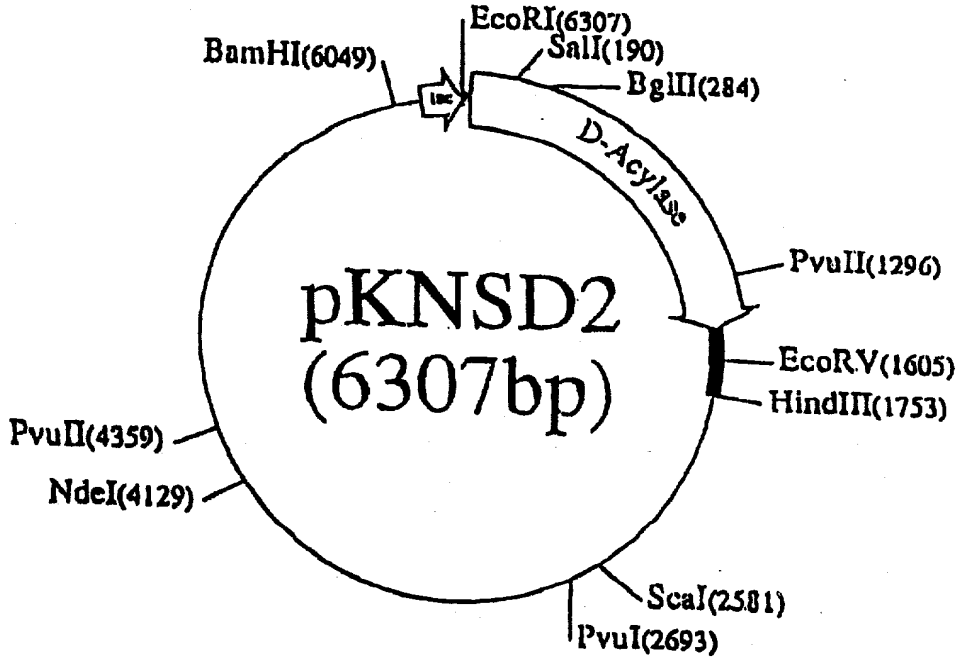
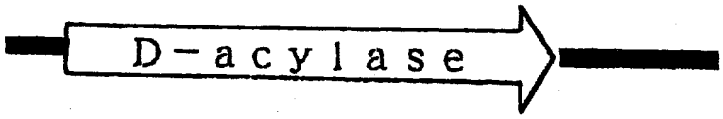
FIG. 2



insert fragment : 

Declaration and Power of Attorney For Patent Application

特許出願宣言書及び委任状

Japanese Language Declaration

日本語宣言書

下記の氏名の発明者として、私は以下の通り宣言します。

As a below named inventor, I hereby declare that:

私の住所、私書箱、国籍は下記の私の氏名の後に記載された通りです。

My residence, post office address and citizenship are as stated next to my name.

下記の名称の発明に関して請求範囲に記載され、特許出願している発明内容について、私が最初かつ唯一の発明者（下記の氏名が一つの場合）もしくは最初かつ共同発明者（下記の名称が複数の場合）であると信じています。

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled.

TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING D-AMINOACYLASE

上記発明の明細書は、

☐ 本書に添付されています。

☐ ___月___日に提出され、米国出願番号または特許協定条約国際出願番号を_____とし、
（該当する場合）_____に訂正されました。

the specification of which

☐ is attached hereto.

☒ was filed on June 15, 2000
as United States Application Number or
PCT International Application Number
PCT/JP00/03932 and was amended on
_____ (if applicable).

私は、特許請求範囲を含む上記訂正後の明細書を検討し、内容を理解していることをここに表明します。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37編第1条56項に定義されるとおり、特許資格の有無について重要な情報を開示する義務があることを認めます。

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

Japanese Language Declaration
(日本語宣言書)

私は、米国法典第35編119条（a）-（d）項又は365条（b）項に基づき下記の、米国以外の国の少なくとも一ヵ国を指定している特許協力条約365（a）項に基づく国際出願、又は外国での特許出願もしくは発明者証の出願についての外国優先権をここに主張するとともに、優先権を主張している、本出願の前に出願された特許または発明者証の外国出願を以下に、枠内をマークすることで、示しています。

I hereby claim foreign priority under Title 35, United States Code, Section 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s) 外国での先行出願			Priority Claimed 優先権主張	
11-170555	Japan	17/June/1999	☒ Yes はい	☐ No いいえ
(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)		
			☐ Yes はい	☐ No いいえ
(Number) (番号)	(Country) (国名)	(Day/Month/Year Filed) (出願年月日)		

私は、第35編米国法典119条（e）項に基づいて下記の米国特許出願規定に記載された権利をここに主張いたします。

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

(Application No.) (出願番号)	(Filing Date) (出願日)

私は、下記の米国法典第35編120条に基づいて下記の米国特許出願に記載された権利、又は米国を指定している特許協力条約365条（c）に基づく権利をここに主張します。また、本出願の各請求範囲の内容が米国法典第35編112条第1項又は特許協力条約で規定された方法で先行する米国特許出願に開示されていない限り、その先行米国出願書提出日以降で本出願書の日本国内または特許協力条約国際提出日までの期間中に入手された、連邦規則法典第37編1条56項で定義された特許資格の有無に関する重要な情報について開示義務があることを認識しています。

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of application.

(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)
(Application No.) (出願番号)	(Filing Date) (出願日)	(Status: Patented, Pending, Abandoned) (現況：特許許可済、係属中、放棄済)

私は、私自信の知識に基づいて本宣言書中で私が行なう表明が真実であり、かつ私の入手した情報と私の信じるところに基づく表明が全て真実であると信じていること、さらに故意になされた虚偽の表明及びそれと同等の行為は米国法典第18編第1001条に基づき、罰金または拘禁、もしくはその両方により処罰されること、そしてそのような故意による虚偽の声明を行なえば、出願した、又は既に許可された特許の有効性が失われることを認識し、よってここに上記のごとく宣誓を致します。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration
(日本語宣言書)

委任状：私は下記の発明者として、本出願に関する一切の手続きを米特許商標局に対して遂行する弁理士または代理人として、下記の者を指名いたします。
(弁護士、または代理人の指名及び登録番号を明記のこと)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: *(list name and registration number)*

書類送付先

Send Correspondence to:

直接電話連絡先：（名前及び電話番号）

Direct Telephone Calls to: (name and telephone number)

(703) 413-3000

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国籍	Citizenship Japan
郵便の宛先	Post Office Address c/o Amano Enzyme Inc. Gifu R & D Center, 4-179-35, Sue-cho, Kakamigahara-shi, Gifu 509-0108 JAPAN
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第二の共同発明者の署名　日付	Second joint Inventor's signature　Date Yoshinao Koide　Jan. 25, 2002
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国籍	Citizenship Japan
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(第三以降の共同発明者についても同様に記載し、署名すること)

(Supply similar information and signature for third and subsequent joint inventors.)

Japanese Language Declaration
(日本語宣言書)

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第三の共同発明者の署名 日付	Third joint Inventor's signature Date Yoshihiko Hirose Jan. 25, 2002
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国籍	Citizenship Japan
郵便の宛先	Post Office Address c/o Amano Enzyme Inc. ~~Gifu R & D Center, 4-179-35,~~ Sue-cho, Kakamigahara-shi, Gifu ~~509-0108 JAPAN~~

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第四の共同発明者の署名 日付	Fourth joint Inventor's signature Date Jan. 25, 2002
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国籍	Citizenship Japan
郵便の宛先	Post Office Address 700, Oaza Tannohara, Oita-shi, OITA 870-1124 JAPAN

第五の共同発明者の氏名 5-00	Full name of fifth joint inventor, if any Kimiyasu ISOBE
第五の共同発明者の署名 日付	Fifth joint Inventor's signature Date Jan. 25, 2002
住所	Residence Iwate, Japan JPX
国籍	Citizenship Japan
郵便の宛先	Post Office Address 3-15-40, Kuroishino, Morioka-shi, IWATE 020-0111 JAPAN

第六の共同発明者の氏名	Full name of sixth joint inventor, if any
第六の共同発明者の署名 日付	Sixth joint Inventor's signature Date
住所	Residence
国籍	Citizenship
郵便の宛先	Post Office Address

(第六またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。)

(Supply similar information and signature for third and subsequent joint inventors.)

SEQUENCE LISTING

<110> TAKEUCHI, Ken-ichi
KOIDE, Yoshinao
HIROSE, Yoshihiko
MORIGUCHI, Mitsuaki
ISOBE, Kimiyasu

<120> TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING D-AMINOACYLASE

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Ala Thr Pro Ala Ala Val Asn Ala Ala Cys Met Val Gly His Ser Thr
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Ile Asp Ser His Thr His Asp Asp Asn Tyr Leu Leu Arg Arg Arg Asp
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Met Thr Pro Lys Ile Ser Gln Gly Val Thr Thr Val Val Thr Gly Asn
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Cys Gly Ile Ser Leu Ala Pro Leu Ala His Ala Asn Pro Pro Ala Pro
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(IPOK00-010 WOUS)

~~配列表~~

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↳ (name changed) Amano Enzyme Inc.

<120> 形質転換微生物、D－アミノアシラーゼの製造方法

↳ Transformed Microorganism and Process for Producing D-aminoacylase

<130> POK-99-022

↳ IPOK00-010WOUS

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gaattccact tgatcgcgga aggagagatt tcc atg tcc caa tcc gat tcc cag ccc  57
                                     Met Ser Gln Ser Asp Ser Gln Pro
                                     1               5
ttc gac ctg ctg ctc gcg ggc ggc acc ctc atc gac ggc agc aac acc       105
Phe Asp Leu Leu Leu Ala Gly Gly Thr Leu Ile Asp Gly Ser Asn Thr
    10                  15                  20
ccg ggg cgg cgc gcc gac ctg ggc gtg cgc ggc gac cgc atc gcc gcc       153
Pro Gly Arg Arg Ala Asp Leu Gly Val Arg Gly Asp Arg Ile Ala Ala
25                  30                  35                  40
```

atc ggc gat ctg tcg gac gcc gcc gcg cac acc cgg gtc gac gtg tcg 201
Ile Gly Asp Leu Ser Asp Ala Ala Ala His Thr Arg Val Asp Val Ser
45 50 55

ggc ctg gtg gtc gcg ccc ggc ttc atc gac tcg cac acc cac gac gac 249
Gly Leu Val Val Ala Pro Gly Phe Ile Asp Ser His Thr His Asp Asp
60 65 70

aac tac ctg ctc agg cgt cgc gac atg acg ccc aag atc tcg cag ggc 297
Asn Tyr Leu Leu Arg Arg Arg Asp Met Thr Pro Lys Ile Ser Gln Gly
75 80 85

gtc acc acg gtg gtc acg ggc aat tgc ggc atc agc ctg gcg ccg ctg 345
Val Thr Thr Val Val Thr Gly Asn Cys Gly Ile Ser Leu Ala Pro Leu
90 95 100

gcg cac gcc aac ccg ccc gcc ccc ctg gac ctg ctg gac gaa ggc ggc 393
Ala His Ala Asn Pro Pro Ala Pro Leu Asp Leu Leu Asp Glu Gly Gly
105 110 115 120

tct tac cgt ttc gag cgc ttc gcc gac tac ctg gac gcg ttg cgg gcc 441
Ser Tyr Arg Phe Glu Arg Phe Ala Asp Tyr Leu Asp Ala Leu Arg Ala
125 130 135

acg ccg gcg gcc gtc aac gcc gcc tgt atg gtg ggc cat tca acg ctg 489
Thr Pro Ala Ala Val Asn Ala Ala Cys Met Val Gly His Ser Thr Leu
140 145 150

cgc gcc gcg gtc atg ccg gac ttg cag cgc gcc gcc acc gac gag gaa 537
Arg Ala Ala Val Met Pro Asp Leu Gln Arg Ala Ala Thr Asp Glu Glu
155 160 165

atc gcg gcc atg cgg gac ctg gcc gag gaa gcc atg gcc agc ggc gcc 585
Ile Ala Ala Met Arg Asp Leu Ala Glu Glu Ala Met Ala Ser Gly Ala
170 175 180

atc ggc att tcg acc ggc gcc ttc tac ccg ccc gcc gcc cgc gcc acc 633
Ile Gly Ile Ser Thr Gly Ala Phe Tyr Pro Pro Ala Ala Arg Ala Thr
185 190 195 200

acc gaa gag atc atc gag gtg tgc cgg ccg ctg agc gcg cat ggc ggc 681
Thr Glu Glu Ile Ile Glu Val Cys Arg Pro Leu Ser Ala His Gly Gly
205 210 215

atc tac gcc acc cac atg cgc gac gaa ggc gag cac atc gtg gcc gcg 729
Ile Tyr Ala Thr His Met Arg Asp Glu Gly Glu His Ile Val Ala Ala
220 225 230

ctg gag gaa acc ttc cgc atc ggc cgc gag ctg gac gtg ccg gtg gtg 777
Leu Glu Glu Thr Phe Arg Ile Gly Arg Glu Leu Asp Val Pro Val Val
235 240 245

atc tcg cac cac aag gtc atg ggc cag ccc aat ttc ggc cgc tcg cgc 825
Ile Ser His His Lys Val Met Gly Gln Pro Asn Phe Gly Arg Ser Arg
250 255 260

gag acg ctg ccg ctg atc gag gcc gcc atg gcg cgc cag gac gtc tcg 873
Glu Thr Leu Pro Leu Ile Glu Ala Ala Met Ala Arg Gln Asp Val Ser
265 270 275 280

ctg gac gcg tat ccc tac gtg gcc ggc tcc acc atg ctc aag cag gac 921
Leu Asp Ala Tyr Pro Tyr Val Ala Gly Ser Thr Met Leu Lys Gln Asp
285 290 295

cgc gtg ctg ctg gcc gga cgc acc atc atc acc tgg tgc aag ccc ttc 969
Arg Val Leu Leu Ala Gly Arg Thr Ile Ile Thr Trp Cys Lys Pro Phe
300 305 310

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Pro Glu Leu Ser Gly Arg Asp Leu Asp Glu Val Ala Ala Glu Arg Gly
315 320 325

aaa tcc aag tac gac gtg gtg ccc gag ctg cag ccg gcc ggc gcc atc 1065
Lys Ser Lys Tyr Asp Val Val Pro Glu Leu Gln Pro Ala Gly Ala Ile
330 335 340

tac ttc atg atg gac gaa ccc gac gtg cag cgc atc ctg gcg ttc ggc 1113
Tyr Phe Met Met Asp Glu Pro Asp Val Gln Arg Ile Leu Ala Phe Gly
345 350 355 360

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Pro Thr Met Ile Gly Ser Asp Gly Leu Pro His Asp Glu Arg Pro His
365 370 375

ccg cgc ctg tgg ggc acc ttc ccg cgg gtg ctg ggg cac tat gcg cgc 1209
Pro Arg Leu Trp Gly Thr Phe Pro Arg Val Leu Gly His Tyr Ala Arg
380 385 390

gac ctg ggc ctg ttc ccg ctg gag acg gcg gta tgg aag atg acc ggc 1257
Asp Leu Gly Leu Phe Pro Leu Glu Thr Ala Val Trp Lys Met Thr Gly
395 400 405

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Leu Thr Ala Ala Arg Phe Gly Leu Ala Gly Arg Gly Gln Leu Gln Ala
410 415 420

ggg tac ttc gcc gac ctg gtg gtg ttc gac ccg gcc acg gtg gcc gat 1353
Gly Tyr Phe Ala Asp Leu Val Val Phe Asp Pro Ala Thr Val Ala Asp
425 430 435

acc gcc acc ttc gaa cac cct acc gag cgc gcc gcc ggc atc cat tcc 1401
Thr Ala Thr Phe Glu His Pro Thr Glu Arg Ala Ala Gly Ile His Ser
440 445 450 455

gtg tac gtc aac ggc gcg ccg gtc tgg caa gag cag gcg ttc acc ggc 1449
Val Tyr Val Asn Gly Ala Pro Val Trp Gln Glu Gln Ala Phe Thr Gly
460 465 470

cag cat gcc ggc cgc gtg ctc gca cgc acg gcc gcc tg agcccggcgc 1497

Gln His Ala Gly Arg Val Leu Ala Arg Thr Ala Ala

475 480 483

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